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Abstract

Background

Serotonin (5-hydroxytryptamine, 5-HT) has been linked with several inflammation-associated intestinal diseases including ulcerative colitis (UC). The largest pool of 5-HT in the body is in enterochromaffin (EC) cells located throughout the intestinal tract. EC cells are mechanosensitive and detect noxious stimuli, inducing secretion of 5-HT which plays an important role in enteric reflexes and immunomodulation. In this study, we evaluated intestinal 5-HT levels in the *Winnie* mouse model of spontaneous chronic colitis which closely replicates UC.

Methods

Real-time electrochemical recordings of 5-HT oxidation currents were obtained from *ex vivo* preparations of jejunum, ileum, proximal and distal colon from *Winnie* (5-25 weeks old) and age matched C57BL/6 mice. EC cells were examined by immunohistochemistry, and the gene expression of tryptophan hydroxylase 1 (5-HT synthesis) and the serotonin reuptake transporter (SERT) were determined by quantitative Real-Time Polymerase Chain Reaction (RT-qPCR).

Results

Compression-evoked, and basal 5-HT concentrations were elevated in the distal and proximal colon of *Winnie* mice. EC cell hyperplasia and downregulation of SERT on the transcriptional level were identified as mechanisms underlying increased levels of 5-HT. Increase in mucosal 5-HT release was observed at the onset of disease at 7-14 weeks, confirmed by disease activity scores. Furthermore, increases in 5-HT levels and progression of disease activity correlated linearly with age but not sex.

Conclusions

Our findings in the *Winnie* mouse model of spontaneous chronic colitis demonstrate for the first time that the onset and progression of chronic UC-like intestinal inflammation is associated with increased 5-HT levels in the colonic mucosa.

Key words: 5-HT, Serotonin, *Winnie* mouse model, IBD, Ulcerative colitis

Introduction

Inflammatory bowel disease (IBD) is a chronic disorder consisting of two main pathologies: Crohn's disease (CD) and ulcerative colitis (UC). Primarily these diseases are distinguished by severe chronic inflammation observed as transmural skip lesions throughout the intestinal tract in CD, while in UC the mucosal and submucosal inflammation continuously ascends from the rectum to colon. IBD manifestations and sequela include diarrhoea and/or constipation, ulceration, strictures, the formation of fistulae and intense abdominal pain (1). Due to the idiopathic nature of IBD there is no cure; thus, relief from its debilitating symptoms is paramount. Managing IBD over a long period is difficult due to either the toxicity of therapies, or refractory responses in patients (2). The inefficacy of current treatments are highlighted by the bowel resection rate in up to 90% of CD patients (3). Investigation of endogenous biochemical signals contributing to chronic intestinal inflammation may lead to the development of more effective therapies.

Serotonin (5-hydroxytryptamine, 5-HT) has long been linked with intestinal diseases including CD, UC, irritable bowel syndrome (IBS), coeliac disease and diverticulitis (4). The largest pool of 5-HT in the body is secreted by a specialised subset of enteroendocrine cells called enterochromaffin (EC) cells located throughout the intestinal tract (5). EC cells produce 5-HT from exogenous tryptophan via tryptophan hydroxylase 1 (Tph1) and package it into vesicles for organised release. Subsequently, 5-HT acts as an agonist for a variety of 5-HT receptors; these actions conclude by the uptake of 5-HT into epithelial cells by the serotonin reuptake transporter (SERT) where it is metabolised via monoamine oxidase A (6). 5-HT release from EC cells is mechanosensitive (7), albeit EC cells possess a variety of receptors that detect nutrients and noxious stimuli including pro-inflammatory mediators,

bacterial metabolites and chemical irritants that induce 5-HT secretion (6, 8-10). Once released, 5-HT acts in a paracrine manner stimulating both intrinsic afferent nerve terminals that regulate peristaltic reflexes (11), and extrinsic afferent nerve terminals transmitting sensory information via vagal and spinal pathways (12). Thus increased 5-HT release has been associated with altered motility and visceral discomfort in intestinal inflammation. Furthermore, 5-HT alters the functions of innate leukocytes including monocytes, macrophages, dendritic cells, T and B-lymphocytes (13). 5-HT may also have chemotactic properties on leukocytes as observed in eosinophils and dendritic cells (14, 15).

Increased 5-HT availability has been reported in animal models of chemically-induced acute intestinal inflammation (16-20). The association between 5-HT and inflammation has been further demonstrated by reduced severity of colitis in Tph1 null mice (21). The significance of 5-HT in the inflamed gut has not been determined in a chronic model replicating the disease course of human IBD. *Winnie* mice with spontaneous chronic colitis are an ideal model of IBD due to its close pathophysiological resemblance to human UC (22-26). These mice have a single point missense mutation in the *Muc2* gene causing aberrant assembly of mucin, resulting in epithelial barrier dysfunction, and Th17 (CD4+ and interleukin 17+ T-cell)-type chronic inflammation leading to UC-like symptoms including altered gastrointestinal transit, motility and chronic diarrhoea. The objectives of this study were to define whether the levels of mucosa-derived 5-HT are changed in *Winnie* mice, elucidate mechanisms underlying any changes, and determine whether 5-HT levels are associated with the onset and progression of chronic colitis.

Methods

Animals

Male and female *Winnie* mice aged 14-16 weeks (total n=13) were obtained from the University of Tasmania (Launceston, Tasmania, Australia) and Victoria University (Melbourne, Victoria, Australia) for 5-HT measurements, immunohistochemistry and quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) studies. *Winnie* mice were compared to aged matched male and female C57BL/6 mice (total n=10) obtained from the Animal Resource Centre (Perth, Western Australia, Australia). For disease onset and progression studies, male and female *Winnie* mice aged 5-6, 7-14, 15-19 and 20-25 weeks (total n=24) were obtained from the University of Tasmania (Launceston, Tasmania, Australia) and Victoria University (Melbourne, Victoria, Australia). All mice had access to food and water *ad libitum*, and were housed in a temperature-controlled environment with a 12-h day/night cycle. All mice were acclimatised for at least one week at the Western Centre for Health, Research and Education (Melbourne, Victoria, Australia) to reduce the environmental impact on intestinal health. Mice were killed by cervical dislocation and tissues were collected for subsequent *ex vivo* procedures. In humans, differential effects of 5-HT on the distal and proximal colon have been reported (27). Furthermore, differences in colitis are observed between the distal colon and proximal colon in *Winnie* mice (25, 26); thus these tissues were separated for all subsequent experiments and analysis. All animal experiments in this study complied with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Victoria University Animal Experimentation Ethics Committee.

5-HT measurements

Intestinal 5-HT was measured using an electrochemical technique previously validated in multiple species including mice (16, 28-30). Segments of the jejunum, ileum, proximal and distal colon were visualised under a dissecting microscope, cut along the mesenteric border

and loosely pinned mucosal side up in a silicon-lined recording chamber. The chamber was superfused with carbogen (95% O2 and 5% CO2) bubbled physiological Krebs solution (composition in mmol L^{-1}: NaCl, 117; NaH2PO4, 1.2; MgSO4, 1.2; CaCl2, 2.5; KCl, 4.7; NaHCO3, 25; and glucose, 11) at 35°C at a flow rate of ~5mL/min. Tissues were equilibrated for 60 min before amperometric recordings of 5-HT oxidation commenced. Microelectrodes were prepared by insulating a 7μm carbon fibre with a borosilicate glass capillary (outer diameter, 1.5-mm; inner diameter, 0.86-mm; Harvard Apparatus, Holliston, MA, USA) leaving ~200μm of carbon fibre exposed at the recording tip. Within the capillary, a pellet of woods metal was used to join the remaining carbon fibre and copper wire to provide a connection point for the head-stage. Carbon fibre electrodes were voltage clamped at +400 mV; 5-HT oxidation was detected as a positive current deflection. Recordings of the current generated by the oxidation of 5-HT were made using a VA-10 amplifier (NPI Electronics, Tamm, Germany), digitized at 1–5 kHz (Digidata 1440; Axon Instruments, Union City, CA, USA) to a personal computer using PClamp 9.0 (MDS Analytical Technologies, Mississauga, ON, Canada, 0.5 kHz filtering with a 50-Hz notch filter). All manufactured electrodes were individually calibrated with a 10μL spritz of 10μM serotonin hydrochloride (Sigma-Aldrich, Sydney, Australia) in Krebs solution prior to performing recordings. A precision micromanipulator was used to compress the mucosa with the carbon fibre microelectrode to induce mechanically stimulated 5-HT release (peak) and the decay of 5-HT back to baseline levels (steady state).

Immunohistochemistry

Colonic segments were viewed under a dissection microscope, cut along the mesenteric border and pinned mucosal side up in a silicon lined Petri dish (31). Tissues were fixed in 4% paraformaldehyde and 3% sucrose in 0.1 M phosphate buffered saline (PBS) for 4h at 4°C.

Tissues were permeabilised in dimethyl sulfoxide (DMSO) (3x10 min) and washed with 0.1 M PBS (3x10 min) before embedding in optimal cutting temperature (OCT) compound (Tissue Tek-Sakura, Tokyo, Japan). Tissues were cut into 12μm sections using a cryostat and were mounted onto glass slides. OCT sections were allowed to thaw before incubation with 10% normal donkey serum (NDS) and 0.5% Triton X-100 diluted in 0.1 M PBS at room temperature to prevent nonspecific binding with subsequent immunolabeling. Sections were washed as described above, and incubated with rabbit anti-5-HT antiserum (1:5000; Immunostar, Hudson, WI, USA) and 2% NDS overnight at 4°C. Sections were washed as previous, and incubated with donkey anti-rabbit IgG AlexaFluor 647 (Jackson Immunoresearch, West Grove, PA, USA) and 2% NDS for 1 h at room temperature before being counterstained with the nuclei marker, 4',6-diamidino-2-phenylindole (DAPI) for 2 min. Sections were washed prior to being mounted and coverslipped onto glass slides for imaging using DAKO mounting medium (Agilent Technologies, Melbourne, Australia).

Imaging and analysis

Immunoreactivity for 5-HT and DAPI staining was visualised using an Eclipse Ti confocal laser scanning system (Nikon, Tokyo, Japan). Z-series images were acquired using the 40X objective at a nominal thickness of 1μm (512x512 pixels). 5-HT immunoreactivity was pseudo-coloured green for greater visual distinction against DAPI. Only strongly labelled 5-HT positive cells located within the epithelial layer were considered EC cells. The number of EC cells and the number of crypts were quantified in six nonadjacent fields totalling ~4mm^2 per individual sample. From this, the average number of EC cells per crypt was calculated as previously described (16). The height and width of individual crypts were recorded using Image J software (National Institute of Health, Bethesda, MD, USA). For crypt height, 5

measurements were recorded per section in 6 non-adjacent sections per sample (total 30 measurements/sample). For crypt width, 10 measurements were recorded per section in 6 non-adjacent sections per sample (total 60 measurements/sample).

Quantitative Real-Time PCR

Total RNA was extracted from fresh frozen distal colons using TRIzol™ (Thermo Fisher Scientific: Invitrogen, Melbourne, Australia) and purified using an RNeasy® Mini kit (Qiagen, Melbourne, Australia). RNA was treated with DNase (on-column) for 15 min to remove residual genomic DNA. RNA integrity was assessed with an Agilent 2100 Bioanalyzer using Eukaryotic RNA 6000 Nano chips (Agilent Technologies). Only samples with RNA Integrity Numbers (RIN) of greater than 7.9 were included in the study. Total DNase-treated RNA (750ng) from C57BL/6: n=4 animals/group, *Winnie*: n=6 animals/group) was denatured for 5 min at 65°C and reverse transcribed for 1 h at 50°C using Superscript III Reverse Transcriptase (Thermo Fisher Scientific: Invitrogen). The cDNA synthesis reaction was performed in a 20µL reaction volume containing 50mM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$, 0.5mM dNTP, 5mM DTT, 40U RNaseOUT, 0.5µg oligo$(dT)_{12\text{-}18}$ and 200U Superscript III Reverse Transcriptase. The resulting cDNA was diluted 1/25 with nuclease-free water and used for quantitative PCR. The mRNA levels of *SERT* (*Slc6a4*), *Tph1* and reference genes *villin 1* (*vil1*) and *Gapdh*, were detected using PrimePCR™ Assays (Bio-Rad, Sydney, Australia) which comprise pre-designed and validated primer pairs specific for each gene. Descriptions of these assays are provided according to the guidelines proposed by Bustin, *et al.* (32) (Table 1). Amplification conditions were 95°C for a 2 min cycle followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. RT-qPCR was performed in a Bio-Rad CFX96 real-time thermal cycler in a reaction volume of 20µL consisting of 10µL 2×SsoAdvanced™ SYBR® Green supermix (Bio-Rad), 4µL diluted cDNA template, 1µL

gene-specific primer pair (PrimePCR™ Assay, Bio-Rad) and 5µL nuclease-free water. The data was processed using the Bio-Rad CFX Manager™ software (version 3.1), using a constant threshold level to determine crossing point (Ct) values. Four technical replicates were included per sample.

Disease activity index

Colitis is observed in *Winnie* mice by changes to colon weight and body weight, diarrhoea, rectal prolapses and rectal bleeding (25, 26). Colitis was confirmed by a disease activity index (DAI) which included symptoms of chronic diarrhoea (faecal water content: 60-64%=1, 65-69%=2, 70-74%=3, 75-79%=4, ≥80%=5), rectal manifestations (bleeding=1, prolapse=2), weight loss (from highest recorded weight: 1-4%=1, 5-9%=2, ≥10%=3), and ratios of colon weight:length from the caecum to the anus (0.0110-0.0140=1, 0.0141-0.0160=2, 0.0161-0.0180=3, 0.0181-0.0200=4, ≥0.0200=5).

Statistical analysis

Data analysis was performed using GraphPad Prism v7 (GraphPad Software Inc., San Diego, CA, USA). For direct comparisons, data were analysed using Student's *t*-test (two-tailed). X, Y correlations were determined using a linear regression analysis with *P*-values for significant slope relationships recorded. For multiple groups, a one-way ANOVA was performed with post hoc test including the Holm-Sidak method for multiple comparisons and analysis for linear trends. For all analyses $P \leq 0.05$ was considered significant. All data were presented as mean ± standard error of the mean (SEM). For RT-qPCR data, relative gene expression values were calculated relative to one of two reference genes (*villin 1* or *Gapdh*) as -ΔCt values, where $\Delta Ct = Ct_{target} - Ct_{reference}$. Fold expression was calculated by the 2^{-}

$^{\Delta\Delta Ct}$ method (33). Statistical analysis was performed using Student's *t*-test (two-tailed) between -ΔCt values (34). $P \leq 0.05$ was considered significant.

Results

Winnie mice with chronic colitis exhibit increased release of 5-HT from the mucosa of the distal and proximal colon

All *Winnie* mice had severe colitis confirmed by symptoms of chronic diarrhoea, rectal bleeding, lack of weight gain and increased colon weight:length ratios. Electrochemistry was used to measure extracellular 5-HT oxidation currents spatially and temporally in the colonic mucosa of C57BL/6 and *Winnie* mice (Figure 1). The carbon fibre recording electrode was used to quickly compress EC cells in the mucosal crypts which momentarily stimulated 5-HT release (peak) before returning to basal levels (steady state) in the distal (Figure 1A) and proximal (Figure 1B) colon. Quantification of 5-HT in the distal colon revealed a significant elevation of peak and steady state levels in *Winnie* (n=7) mice (peak: 20.8±1.9μM, steady state: 10.2±1.0μM) compared to C57BL/6 (n=5) controls (peak: 9.1±1.6μM, steady state: 4.6±0.7μM, $P<0.01$ for both) (Figure 1A′). Similar results were observed in the proximal colon with peak and steady state 5-HT concentrations approximately double in *Winnie* (n=7) mice (peak: 19.8±2.9μM, steady state: 12.5±2.3μM) compared to C57BL/6 (n=5) controls (peak: 10.2±2.3, steady state: 5.3±1.2μM, $P<0.05$ for both) (Figure 1B′). No differences were observed between the distal and proximal colon for peak and steady state concentrations in C57BL/6 or *Winnie* mice. No differences were observed in 5-HT levels in the ileum and jejunum between *Winnie* and C57BL/6 mice (data not shown).

Enterochromaffin cell hyperplasia correlates with the hypersecretion of 5-HT in the chronically inflamed distal and proximal colon

To determine the cause of increased 5-HT levels in *Winnie* mice, EC cells were quantified by immunohistochemical labelling of vesicular 5-HT stored in the distal and proximal colon (Figure 2A-E‴). EC cell numbers in the distal colon were almost two-fold higher in *Winnie* mice (1.9±0.1 EC cells/crypt) compared to C57BL/6 mice (1.1±0.1 EC cells/crypt, $P<0.001$) (Figure 2E; n=5 animals/group). Likewise, EC cell numbers were elevated in the crypts of the proximal colon in *Winnie* mice (1.9±0.3 EC cells/crypt) compared to C57BL/6 mice (1.0±0.1 EC cells/crypt, $P<0.05$) (Figure 2F; n=5 animals/group). A linear regression analysis was performed to determine if a relationship between 5-HT levels in the distal and proximal colon correlated with EC cell numbers per crypt in C57BL/6 and *Winnie* mice (n=5 animals/group). In the distal colon a significant regression equation was found for the peak ($F(1,8) = 71.01$, $P<0.0001$) and steady state ($F(1,8) = 63.26$, $P<0.0001$) of 5-HT measurements with an R^2 of 0.8987 and 0.8877, respectively (Supplementary Figure 1A-B). Predicted 5-HT concentrations (µM) in the distal colon were equal to -7.60+(15.45×no. of EC cells) for peak responses and -3.32+(7.44×no. of EC cells) for steady state levels. Similarly in the proximal colon, a linear regression analysis revealed a significant equation for the peak ($F(1,8) = 34.82$, $P<0.001$) and steady state ($F(1,8) = 35.45$, $P<0.001$) of 5-HT measurements with an R^2 of 0.8132 and 0.8159, respectively (Supplementary Figure 1C-D; n=5 animals/group). Linear regression equations predicted 5-HT concentrations (µM) to be equal to 0.2+(10.03×no. of EC cells) for peak responses and -0.39+(6.32×no. of EC cells) for steady state levels in the proximal colon.

Crypt morphology shows changes consistent with inflammation in distal and proximal colon of Winnie mice

Alterations to colonic morphology, including crypt height and width, are sensitive metrics of the severity of colonic inflammation in mouse models of inflammation (35). Morphology of

the colonic crypts was analysed in the distal (Figure 3A-B) and proximal (Figure 3C-D) colon of C57BL/6 and *Winnie* mice. In the distal colon, crypts were more elongated in *Winnie* mice (564.2±64.5µm) than C57BL/6 (196.1±13.5µm, $P<0.0001$) (Figure 3E; n=5 animals/group). In the proximal colon the crypts of *Winnie* mice (242.6±36.5µm) were shorter than in the distal colon, however, were still significantly longer than those in the proximal colon from C57BL/6 mice (147.9±17.2µm, $P<0.05$) (Figure 3G). Similarly, measurements of crypt widths revealed significant difference ($P<0.05$) in the *Winnie* distal colon (28.70±1.86µm) compared to C57BL/6 mice (21.46±0.73µm) (Figure 3F; n=5 animals/group) with comparable values obtained in the proximal colon of *Winnie* mice (28.04±1.30µm) and C57BL/6 (20.97±1.05µm, $P<0.01$) (Figure 3H; n=5 animals/group).

Epithelial cell hyperplasia is not specific to enterochromaffin cells in the colon of Winnie mice

Measuring the lengths of the crypts provides a robust indication of epithelial cell hyperplasia originating from the stem cell pool (36). EC cells originate from the same stem cell niche as all epithelial cells at the base of the crypts (37). A linear regression analysis was performed to determine whether the increase in EC cell numbers was due to epithelial hyperplasia as determined by crypt length in the distal and proximal colon. A significant regression equation was found in the distal colon ($F(1,8) = 33.46$, $P<0.001$) and proximal colon ($F(1,8) = 45.52$, $P<0.0001$), with an R^2 of 0.8070 and R^2 of 0.8505, respectively (Supplementary Figure 2A-B; n=5 animals/group). This suggests that the observed increase in EC cell counts per crypt may have been related to the general hyperplasia of epithelial cells.

5-HT levels per quantity of EC cells are disproportionately low in the colon of Winnie mice

Considering our observation of EC cell hyperplasia, we predicted that if changes to EC cell numbers were solely responsible for the increase in 5-HT concentrations then individual EC cells from *Winnie* and C57BL/6 mice would release equal quantities of 5-HT. This was assessed using ratios of electrochemical 5-HT measurements per number of EC cells/crypt measured by immunohistochemistry in colon samples from the same mouse (Figure 4). Ratios of 5-HT:EC cells were significantly higher in the distal colon of *Winnie* mice for both peak and steady state responses (peak: 11.6±0.7, steady state: 5.9±0.4) compared to C57BL/6 (peak: 8.1±0.9, $P<0.05$; steady state: 4.0±0.3, $P<0.01$) suggesting there may be alterations in 5-HT synthesis and/or reuptake in the *Winnie* distal colon (Figure 4A-B; n=5 animals/group). No differences were found in the ratios of 5-HT:EC cells in the proximal colon for peak (Figure 4C) and steady state (Figure 4D) measurements between *Winnie* (peak: 10.1±0.5, steady state: 6.5±0.5) and C57BL/6 (peak: 10.2±2.1, steady state: 5.3±1.0) mice (n=5 animals/group).

Decreased 5-HT reuptake contributes to increased 5-HT levels in the colon of Winnie mice

To determine whether the altered ratio of 5-HT availability per EC cell in the distal colon between C57BL/6 and *Winnie* mice was due to inhibition of 5-HT uptake or increased 5-HT synthesis, mRNA expression of *SERT* (5-HT uptake) and *Tph1* (5-HT synthesis) was investigated using RT-qPCR (Figure 5). In *Winnie* mice, *SERT* was downregulated over two-fold compared to C57BL/6 relative to both *Gapdh* ($2^{-\Delta\Delta Ct} = 0.36$; $P=0.04$) and *villin 1* ($2^{-\Delta\Delta Ct} = 0.36$; $P=0.01$) (Figure 5A-B,E). There was no significant difference in expression of *Tph1* gene expression between C57BL/6 (n=4) and *Winnie* (n=6) mice relative to either *Gapdh* or *villin 1* (Figure 5C-D,E).

Changes to 5-HT levels in Winnie mice are affected by age, not gender, and parallel the changes in severity of colitis

The progression of colitis was observed in *Winnie* mice aged 5 to 25 weeks by a disease activity index (DAI) which included symptoms of chronic diarrhoea, rectal bleeding, lack of weight gain and increased colon weight:length ratios (Figure 6A). Onset of disease was observed at 7-14 weeks (DAI 5.4±0.5) when compared to mice aged 5-6 weeks (DAI 0.2±0.2, $P<0.0001$). By 15-19 weeks, *Winnie* mice exhibit significantly increased disease activity (DAI 8.4±0.5) compared to mice aged 5-6 weeks ($P<0.0001$) and 7-14 weeks ($P<0.01$). Similar observations were made in mice aged 20-25 weeks with the disease activity increased (DAI 10.4±0.8) compared to those aged 5-6 and 7-14 weeks ($P<0.0001$ for both) as well as to mice aged 15-19 weeks ($P<0.05$) (Figure 6A; n=5 animals/group). Post hoc analysis for linear trends revealed a significant relationship between disease activity scores and these age groups ($F(1,16) = 185.1$, $P<0.0001$) with an $R^2_{alerting}$ of 0.9555.

At the same time points, peak and steady state 5-HT release was determined by electrochemical recordings at the mucosal surface of the distal colon in *Winnie* mice (Figure 6B). Peak and steady state 5-HT levels were elevated as early as the 7-14 week (n=8) time point (peak: 15.1±1.0µM, steady state: 8.8±1.2µM) compared to 5-6 week (n=7) old mice (peak: 8.15±0.7µM, steady state: 4.1±0.7µM, $P<0.05$ for both). These results were consistent between 5-6 week old mice and the latter time points of 15-19 weeks (n=9) (peak: 21.3±2.2µM, steady state: 10.4±1.2µM) and 20-25 weeks (n=10) (peak: 21.1±1.4µM, steady state: 11.3±1.0µM) which displayed an elevation in both peak (5-6 vs 15-19 weeks, $P<0.0001$; 5-6 vs 20-25 weeks, $P<0.0001$) and steady state (5-6 vs 15-19 weeks, $P<0.01$; 5-6 vs 20-25 weeks, $P<0.001$) 5-HT levels. In addition, increased peak 5-HT levels were observed in *Winnie* mice aged 15-19 and 20-25 weeks compared to those aged 7-14 weeks ($P<0.05$ for both); however no difference was observed between the 15-19 and 20-25 week

time points. Paralleling our observations with disease activity, post hoc tests suggested 5-HT release was linearly correlated with age for peak ($F(1, 30) = 33.43$, $P<0.0001$) and steady state 5-HT levels ($F(1, 30) = 23.29$, $P<0.0001$) with a $R^2_{alerting}$ value of 0.9201 and 0.9308, respectively.

Previously it was shown that serum levels of mucosa-derived 5-HT are influenced by gender in equines (38); therefore the impact of gender on mucosal 5-HT release in colitis was investigated (Figure 6C). 5-HT concentrations obtained from male and female *Winnie* mice aged 15-25 weeks were compared as this age group provided the most prominent hypersecretion of 5-HT in our study. No significant differences were observed in male (n=9) and female (n=10) *Winnie* mice for peak (male 23.6±2.2µM, female 21.0±1.4 µM) or steady state (male: 11.5±1.3µM, female: 10.9±1.1µM) 5-HT levels.

Discussion

In this study we observed increased basal and mechanically stimulated 5-HT availability in the distal and proximal colon of *Winnie* mice with spontaneous chronic colitis. Correlation analysis revealed that this paralleled an increase in EC cells in the crypts of *Winnie* mice. Crypt hyperplasia, as observed through an increase in crypt length and width in the distal and proximal colon of *Winnie* mice, strongly correlated with an increase in EC cells. However, increased 5-HT availability was not proportional to the number of EC cells alone in the distal colon of *Winnie* mice. Our study revealed that altered 5-HT reuptake due to the downregulation of SERT, together with EC cell hyperplasia, are the underlying mechanisms of increased 5-HT availability in *Winnie* mice. Furthermore, this was the first study to determine intestinal 5-HT release throughout the onset and progression of chronic colitis. We

demonstrated that the hypersecretion of 5-HT parallels the disease severity during its progression which was independent of gender.

Winnie mice used in this study offer several advantages as a model of UC. Like patients with UC, these mice begin to develop spontaneous, chronic inflammation in the colon at early adulthood similar to humans (39, 40). Furthermore, the resemblance in symptoms indicates that similar perturbations may ensue on the cellular and molecular level. Indeed the inflammatory cytokine profile and leukocyte cell populations reflect changes observed in UC patients (25, 26). The similarities between *Winnie* mice and UC patients have been further confirmed by our group by determining alterations in colon morphology and neural innervation, myenteric neuronal damage, changes to colonic motility and transit time as well as faecal microbial and metabolomic profiles (22-24, 41). Together these studies demonstrate that *Winnie* mice provide a robust model of UC. The beginning of disease at 7 weeks of age and chronic ongoing inflammation of the colon in *Winnie* mice permits long-term studies mimicking the onset and progression of UC. Previously, 5-HT levels have been measured in several models of chemically-induced colitis in guinea-pigs, rats and mice (16-20). The majority of these studies investigated 5-HT concentrations in the acute models of 2,4,6-trinitrobenzenesulfonic acid (TNBS) or dextran sulphate sodium (DSS)-induced colitis 4 – 7 days after the induction of inflammation. Due to the short timeframe of these studies, the association between long-term chronic inflammation and 5-HT levels was unknown. Using *Winnie* mice, we were well positioned to study mucosal serotonin release throughout the disease course up to ~175 days of age. This facilitated novel findings that connected increased secretion of mucosa-derived 5-HT with the onset and progression of the disease.

In our study we observed greatly elevated 5-HT levels in the distal and proximal colon in response to chronic colitis using amperometric methods. Previous studies in murine DSS and TNBS-induced colitis have demonstrated increased 5-HT levels in tissue homogenates detected by ELISA (19, 20), however no differences in 5-HT secretion where observed in TNBS-treated animals (20). Similarly, studies in tissues from IBD patients assessing 5-HT levels by ELISA have yielded mixed results (8, 42, 43). Using ELISA, 5-HT levels normalised to tissue weight were decreased in biopsies from UC patients and no changes in 5-HT secretion were observed in biopsy supernatants (42). Nonetheless, 5-HT release is greatly increased EC cells isolated from CD patients compared to healthy controls when exposed to lipopolysaccharides and interleukin 1β. Furthermore, a recent comprehensive study with 75 UC patients observed increased plasma 5-HT levels which could not be explained by an association with SERT polymorphisms (44, 45). Considering that 5-HT in the plasma is originally synthesised and released from EC cells, these results may be indicative of increased local 5-HT concentrations in the mucosa which contrasts prior findings. As previously proposed, quantifying 5-HT by ELISA can be confounded by altered mass of the inflamed tissue or mucosal damage leading to crypt loss and ulcerations, all of which are prominent in intestinal inflammation (17, 46). Electrochemical methods of 5-HT quantification have been utilised by many labs (47, 48) and are less likely to be affected by these structural changes because normalisation to weight or reference proteins is not required. This advantage is highlighted in a mouse model of DSS-induced colitis, whereby increases in 5-HT secretion were obvious using amperometric recordings, but not ELISA (16). Another advantage of amperometric 5-HT recordings is the precise quantification of 5-HT concentrations spatially and temporally. This provides accurate measurements of physiological concentrations of 5-HT released by EC cells in resting and stimulated states. Nevertheless, amperometric quantification of 5-HT also poses limitations including the

availability of equipment, trained personnel and slow data collection in comparison to ELISA.

In our study, increases in mucosal 5-HT paralleled the onset of colonic inflammation rather than preceding it; therefore, this is not likely to be a direct cause of colitis. Nonetheless, hypersecretion of 5-HT may contribute to symptoms of IBD and progression of the disease. In a UC flare, the two most prominent features are abdominal pain and diarrhoea, both symptoms are associated with perturbations of the serotonergic system which plays a role in intestinal nociception and motility as demonstrated in IBS patients (49, 50). In IBD, investigations determining mucosa-derived 5-HT levels and clinical implications of these changes are scarce. Due to the commonality in some symptoms, data from IBS patients may offer insight into the role of 5-HT in manifestations of IBD. In diarrhoea-predominant IBS (IBS-d), increased 5-HT levels are observed in the platelets and platelet-deprived plasma with these stores originating from EC cells of the mucosa (51). Interestingly, in the same study, patients with high 5-HT levels exhibited increased abdominal pain and urgency to defecate. In our study, all *Winnie* mice with high mucosal 5-HT release had loose stool. Previously we demonstrated that diarrhoea in *Winnie* mice was associated with colonic dysmotility and changes in intestinal transit that are similar to IBD patients (24). In the current study we demonstrated that both secreted 5-HT levels and disease activity progress linearly with age. This revealed a trend in the extent of 5-HT hypersecretion with the severity of colitis, including faecal water content. Mucosa-derived 5-HT is thought to have an important role in regulating motility (52). Thus, data presented in our study and those in IBS-d patients may indicate that hypersecretion of 5-HT is at least partially responsible for dysmotility and diarrhoea. Taken together this suggests that altered 5-HT signalling may directly influence the onset of chronic diarrhoea observed in UC. However, the parallels between 5-HT

hypersecretion and colonic hypertrophy observed in our study by ratios of colon weight:length and rectal manifestations suggest that 5-HT signalling has a role in the pathophysiology of colonic inflammation and dysmotility (53).

Due to the abundance of 5-HT receptors throughout the intestinal tract, high 5-HT levels may impact many physiological functions of the inflamed intestine; however, 5-HT may also contribute to the inflammation itself. In animal models of colitis, the importance of 5-HT in the inflammatory response has been demonstrated by applying exogenous 5-HT by enema (46, 54) or its precursor 5-hydroxytryptophan subcutaneously (21). In these studies 5-HT exacerbated colitis in rats with TNBS-induced and mice with DSS-induced colitis establishing a strong pro-inflammatory role for 5-HT (21, 46, 54). This is further evidenced by the abolished effects of chemical inducers of colitis in Tph1 null mice (21) and those orally administered with Tph1 inhibitors (55). These studies demonstrate that 5-HT has a clear role in chemically-induced colitis and that this 5-HT is produced by Tph1, however mechanisms instigating this increase remain disputed. Our results demonstrated that downregulation of SERT elevates secreted 5-HT concentrations in chronic colitis as opposed to increased synthesis by Tph1. Similar observations are documented after TNBS and DSS-induced colitis in mice and guinea-pigs (16, 17, 19, 20). Importantly, SERT expression is also reduced in colonic tissues from UC patients on the protein and transcriptional levels (42). The reduction of SERT is observed in the inflamed mucosa; however its downregulation is also noted in the non-inflamed mucosa of UC patients (56). Previously, it was demonstrated that media conditioned by activated T lymphocytes decreases 5-HT uptake in epithelial cells *in vitro* (57). T lymphocytes were found to secrete TNF-α and IFN-γ; application of these specific cytokines decreased SERT expression and 5-HT uptake with additive effects. Re – activated T lymphocytes isolated from *Winnie* mice show a progressive increase in TNF-α

and IFN-γ secretion with disease progression and may explain how SERT was downregulated in the distal colon of *Winnie* mice (26). Previously, it was shown that SERT deletion exacerbates TNBS-colitis in mice, this strengthens the notion of reducing interstitial 5-HT levels to elicit an anti-inflammatory effect (58). Moreover, it must be noted that administration of the serotonin reuptake inhibitors, fluoxetine and fluvoxamine, have been shown to ameliorate DSS and acetic acid-induced colitis, respectively (59, 60). However, these drugs also act on many other transporters and receptors, thus their mechanisms of action are unclear.

In our study, local 5-HT measurements strongly correlated with the number of EC cells per crypt. Increased numbers of EC cells were reported in the colon of mice (16, 19) and rats (18) with DSS-induced colitis and guinea-pigs with TNBS-induced colitis (17), however, no significant difference was observed in TNBS-exposed mice (20). Increases in EC cell numbers are observed in UC patients (61, 62). Contrarily, EC cell numbers are decreased in severe UC but not non-severe UC, this may indicate the gross architectural damage to the colonic mucosa influences cell counts (42). Similar elevations in EC cells numbers have been observed in CD patients (61), which is reportedly present in active CD ileitis (63) but not in non-active intestinal regions (64). It has been demonstrated that in a murine DSS-induced colitis, EC cell hyperplasia is regulated by interleukin 13 (65). Similar to UC, *Winnie* mice are dominated by the Th2 immune response, consequently, interleukin 13 production is increased in leukocytes from the mesenteric lymph nodes (25, 26) which may contribute to EC cell hyperplasia. In our study, the number of EC cells observed per crypt strongly correlated with crypt length, which is viewed as an indirect measurement of intestinal epithelial cell hyperplasia due to apical migration of superfluous cells increasing the length of the crypts (20). Increases to the length of the crypts are regularly observed in models of

chemically-induced colitis (20, 36). In our study, chronic inflammation resulted in increased crypt length in the distal and proximal colon of *Winnie* mice. Increased crypt length has previously been reported in the distal colon and caecum of the *Winnie* mouse; however no differences were observed in the proximal colon (25). Discrepancies in these data may be explained by the older age of *Winnie* mice used in the present study given that colitis exacerbates with age in *Winnie* mice (26) and inflammation progresses proximally in UC (66). Similar to our observations, the proportion of EC cells to epithelial cells did not change in mice with TNBS-induced colitis despite an increase in crypt length and in number of epithelial cells (20). Together these data suggest the increased EC cell numbers were likely a result of general epithelial cell hyperplasia rather than augmented differentiation of EC cells from the epithelial stem cell pool.

All *Winnie* mice with colitis, regardless of gender, exhibited higher 5-HT release compared to control groups. It has previously been demonstrated that 5-HT synthesis is higher in the brain of males compared to females (67). However, differences in 5-HT levels have also been observed in the serum of equines which indicated that potential gender differences also exist in mucosa-derived 5-HT (38). It has long been predicted that 5-HT mediates the physiological and pathophysiological effects of oestrogen (68). However it has only recently been demonstrated that EC cell hyperplasia is present in oestrus female mice compared to both prooestrus females and males (69). Specifically in IBS-d patients, 5-HT levels are increased in both men and women; however, in women this was dependent on the menstrual cycle with 5-HT levels normalising in women with low progesterone/oestrogen levels (70). The menstrual cycle was not investigated in our study, however, these data may explain why 5-HT levels in female *Winnie* mice appeared marginally lower than in males.

In conclusion, our findings in the *Winnie* mouse model of spontaneous chronic colitis demonstrate for the first time that chronic UC-like intestinal inflammation is associated with hypersecretion of 5-HT from the colonic mucosa. Furthermore, changes to mucosal 5-HT levels parallel the onset and severity of intestinal inflammation. We identified two mechanisms that are responsible for increased levels of 5-HT, EC cell hyperplasia in both the distal and proximal colon as well as downregulation of SERT on the transcriptional level in the distal colon. *Winnie* mice may provide a robust model to study the immunomodulatory role of 5-HT in colitis and pre-clinically test pharmaceutical compounds targeting the serotonergic system.

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Conflict of interest

No conflict of interest

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Table 1: Description of PrimePCR™ assay targets.

Gene	Gene symbol	Ensembl Gene ID	Chromosome Location *	Bio-Rad Unique Assay ID	Amplicon Context Sequence	Amplicon length (bp)
Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	SERT Slc6a4 5-HTT	ENSMUSG00000020838	11:77010683-77012930	qMmuCED0045158	CTGGGGCAAGAAGATGGATTTCCTCCTGTCTGTCATTGGCTATGCCGTGGACCTGGGCAACATCTGGCGTTTTCCCTACATATGCTACCAGAATGGTGGAGGGGCCTTCCT	81
Tryptophan hydroxylase 1	Tph1	ENSMUSG00000040046	7:46662129-46665237	qMmuCID0040148	AATTCTGAATTTCTTTGCTTTGATTTCCGGGACTCGATGTGTAACAGGCTCACATGATTCTCCTGGAAGATTTTCAGCACTTTTATGAGTCCTCCGACTTCATTCTCCAAG GAGAAGATGAGAG	94
villin 1	vil1	ENSMUSG00000026175	1:74416009-74418428	qMmuCID0021827	TCTTCGATGGTGACTGCTATGTAGTCCTGGCTATCCACAAGACCAGCAGCACTCTCTCCTATGATATCCACTACTGGATTGGCCAGGACTCGTCCCAGGAT	71
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	ENSMUSG00000057666	6:125162278-125162382	qMmuCED0027497	TGGGAGTTGCTGTTGAAGTCGCAGGAGACAACCTGGTCCTCAGTGTAGCCCAAGATGCCCTTCAGTGGGCCCTCAGATGCCTGCTTCACCACCTTCTTGATGTCA	75

Figure descriptions

Figure 1. *Electrochemical measurements of 5-HT at the mucosal surface of the colon.* 5-HT was measured using an electrochemical technique where carbon fibre electrodes are used to mechanically stimulate the colonic mucosa and oxidise 5-HT when voltage clamped at +400mV generating a current proportional to the temporal and spatial availability of 5-HT. Representative amperometric traces of the current generated by 5-HT oxidisation at the mucosal surface of the **(A)** distal and **(B)** proximal colon. The current (nA) was converted to 5-HT (µM) by calibrating carbon fibre electrodes with 10µM 5-HT solution. Mechanical stimulation of the colonic mucosa (grey bars) produced a compression-evoked (peak) release of 5-HT which decayed back to basal levels (steady state) in both C57BL/6 (grey traces) and *Winnie* (black traces) mice. Dotted lines represent baseline. Comparison of the 'peak' and 'steady state' 5-HT levels in the **(A′)** distal and **(B′)** proximal colon of C57BL/6 and *Winnie* mice. **P*<0.05, ***P*<0.01; six to ten replicates/sample; C57BL/6: n=5 animals/group, *Winnie:* n=7 animals/group.

Figure 2. *Quantification of enterochromaffin (EC) cells in cross sections of the colon.* EC cells were observed using fluorescent immunohistochemical detection of mucosal 5-HT positive (green) cell bodies in the **(A-B′)** distal colon (DC) and **(C-D′)** proximal colon (PC) of **(A,C)** C57BL/6 and **(B-B′ ,D-D′)** *Winnie* mice (Scale bar = 50µm). High magnification images **(B′, D′)** (60X) (Scale bar = 50µm) and serial confocal sections with a Z-step of 0.5 µm **(E-E′′′)** (Scale bar = 10µm) confirmed that 5-HT immunoreactivity (green) originated from nucleated cells (DAPI – blue) within the colonic crypts. Quantification of 5-HT positive EC cells in immunofluorescent images per number of crypts in the **(E)** distal colon and **(F)** proximal colon of C57BL/6 and *Winnie* mice. **P*<0.05, ****P*<0.001; six replicates/sample; n=5 animals/group.

Figure 3. *Crypt morphology in the distal colon and proximal colon.* Representative images of crypt morphology in the (**A-B**) distal colon (DC) and proximal colon (**C-D**) of **(A,C)** C57BL/6 mice and **(B,D)** *Winnie* mice visualised using confocal microscopy of cross sections labelled with DAPI (20X magnification, scale bar = 50μm). These images were used for the quantification of the length of the crypts and the width of the crypts in the (**E-F**) distal colon and (**G-H**) proximal colon of C57BL/6 and *Winnie* mice (note: the crypt length of some *Winnie* mice were quantified in images using the 10X objective to fit within the field of view). *P<0.05, ****P<0.0001; six replicates/sample n=5 animals/group.

Figure 4. *5-HT availability per number of EC cells in the colonic crypts.* Ratios calculated from 5-HT measurements per average number of EC cells observed in each crypt of individual C57BL/6 and *Winnie* mice. Average ratios of 5-HT release per EC cell in the distal colon for **(A)** compression-evoked 5-HT release (peak) and **(B)** basal 5-HT levels (steady state). Average ratios of 5-HT release per EC cell in the proximal colon for **(C)** compression-evoked 5-HT release (peak) and **(D)** basal 5-HT levels (steady state).*P<0.05, **P<0.01; n=5 animals/group.

Figure 5. *Tph1 and SERT mRNA expression in the distal colon.* Box (median, IQR) and whisker (range) representation of *SERT* mRNA expression assessed by RT-qPCR in *Winnie* compared to C57BL/6 mice relative to **A)** *Gapdh* and **B)** *villin 1* reference genes. Box

(median, IQR) and whisker (range) representation of *Tph1* mRNA expression assessed by RT-qPCR in *Winnie* compared to C57BL/6 mice relative to **C)** *Gapdh* and **D)** *villin 1* reference genes. Relative gene expressions are presented as -ΔCt values. Fold expression ($2^{-\Delta\Delta Ct}$) of *Tph1* and *SERT* relative to *Gapdh* and *villin 1* reference genes in *Winnie* mice (**E**) compared to compared to C57BL/6 mice ($2^{-\Delta\Delta Ct}$ = 1.00). **P*<0.05; six replicates/sample; C57BL/6: n=4 animals/group, *Winnie*: n=6 animals/group.

Figure 6. *Effects of age/disease activity and gender on 5-HT availability in the inflamed distal colon of Winnie mice.* **(A)** The progression of colitis assessed by the disease activity index (DAI) in *Winnies* aged 5-6, 7-14, 15-19 and 20-25 weeks. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001; six to ten replicates/sample; n=5 animals/group. **(B)** Electrochemical quantification of compression-evoked (peak) and basal levels (steady state) of 5-HT at the mucosal surface of the colon from *Winnie* mice at various stages of the progression of chronic spontaneous colitis. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001; six to ten replicates/sample; 5-6 weeks: n=7, 7-14 weeks: n=8, 15-19 weeks: n=9, 20-25 weeks: n=10 animals/group. **(C)** Effects of gender on compression-evoked (peak) and basal levels (steady state) of 5-HT in colitis determined by electrochemical quantification. six to ten replicates/sample; male: n=9 and female: n=8 animals/group.

Supplementary Figure 1. *Linear regression analysis of 5-HT measurements and enterochromaffin (EC) cell numbers.* Linear correlations of EC cell numbers per crypt (*x axis)* in the distal colon versus **(A)** compression-evoked 5-HT release (peak, *y axis*) and from the **(B)** basal levels of 5-HT (steady state, *y axis*). Linear correlations of EC cell numbers per crypt (*x axis)* in the proximal colon versus **(C)** compression-evoked 5-HT release (peak, *y axis*) and from the **(D)** basal levels of 5-HT (steady state, *y axis*). Grey dots represent values

from individual C57BL/6 mice and black dots represent values from individual *Winnie* mice; n=5 animals/group.

Supplementary Figure 2. *Linear regression analysis of enterochromaffin cell numbers and crypt lengths in the distal and proximal colon.* Linear correlation of EC cell numbers per crypt (*x axis)* versus the length of crypts (*y axis*) from the **(A)** distal colon and the **(B)** proximal colon. Grey dots represent values from individual C57BL/6 mice and black dots represent values from individual *Winnie* mice; n=5 animals/group.